FACTORS INFLUENCING METAMORPHOSIS OF BUGULA LARVAE

WILLIAM F. LYNCH

St. Ambrose College, Davenport, Iowa, and the Marine Biological Laboratory, Woods Hole, Mass.

A review of the literature on experimentally induced metamorphosis in invertebrates and in sessile tunicates suggests the possibility that essentially similar mechanisms may be involved in animals of widely different phyla. As in parthenogenesis, metamorphosis can be induced experimentally by a wide variety of conditions and chemical agents, generally lacking any semblance of specificity; and most of these seem to be unfavorable to larval life. Huxley (1928) effected metamorphosis in Echinus plutei by treating them with very dilute concentrations of $HgCl_2$ ($M/2,000,000$), and Runnström (1917) found that starvation of the larvae, over-ripeness of the eggs from which they developed and very dilute solutions of $ZnSO_4$ in sea water would cause dedifferentiation in Echinus larvae. Grave (1935) found that ascidian metamorphosis can be accelerated by many agents including CO_2-free sea water, lactic acid, precipitates formed when NaOH, KOH or $BaCl_2$ are added to sea water, whole pituitary gland, tissue extracts of ascidian mantle and endostyle and of the foot and mantle of snails; these extracts are highly specific in nature. Other promoters of ascidian metamorphosis include: copper (Grave, 1941; Bertholf and Mast, 1944; Glaser and Anslow, 1949), concentrated sea water (Bertholf and Mast, 1944), thyroid extract (Weiss, 1928) and whole gland (Grave, 1935), methylene blue (Bertholf, 1945; Zhinkin, 1938), neutral red (Bradway, 1936; Zhinkin, 1938), brilliant cresyl blue (Zhinkin, 1938), Janus green (Bertholf and Mast, 1944), a brief exposure to distilled water (Bertholf, 1945), inorganic iodine but not thyroxine (Bradway, 1936), "conditioned sea water" (Grave and Nicoll, 1936; Grave, 1944; Bertholf and Mast, 1944) and isotonic NaCl (Bradway, 1936). (Grave, 1935, had found that the addition of sodium and potassium ions to sea water had no effect on metamorphosis; Bradway, however, used a different technique with an optimum amount of exposure and used isotonic NaCl instead of sea water to which the ions had been added.) Metamorphosis can be induced in the larva of the oyster, *Ostrea virginica,* by copper (Prytherch, 1934; Armstrong and Miall, 1945) and by a certain optimum of salinity (Prytherch, 1934); but neither the cations nor anions of sea water nor the metal carbonates of Fe, Al, Ag, Ni, Ba, Pb and Sn have any effect according to Prytherch (1934). Copper, hypertonic sea water and heat accelerate metamorphosis in the bryozoan, *Bugula flabellata* (Lynch, 1949a).

Since fluids in which proteolytic digestion has occurred bring about a high concentration of neutral red from very dilute solutions, whereas coelomic fluids do not take the stain, Koehring (1930, 1931) proposed that the azo radical of the dye combines with and activates proteolytic enzymes and that neutral red staining can be used as an index of the site and intensity of enzyme activity. She found the dye

effective in causing parthenogenesis in starfish eggs. Neutral red in concentrations of 1 : 50,000 parts (Bradway, 1936) and of 1 : 200,000–1 : 800,000 parts in sea water (Zhinkin, 1938) accelerates metamorphosis in ascidian larvae, presumably by activating autolytic enzymes involved in resorption of the tail. Zhinkin (1938) found methylene blue also effective in approximately the same concentrations. Since some agents have the common effect of inducing precocious fixation in animals of widely different phyla, it was thought that the two vital dyes might accelerate metamorphosis in *Bugula* larvae.

The present paper concerns a re-investigation of the role played by the ions of sea water under more rigidly controlled conditions of light, osmotic pressure and the pH of the medium than in former experiments, and of the effects of media containing only three or two ions and combinations of these with vital dyes; the work was undertaken since it was considered that certain factors may act antagonistically to accelerators of metamorphosis whereas others may synergize their effects. This supposition was verified, sometimes in a striking manner. The larvae were secured by means already described (Lynch, 1947) and the methods used will be presented in the separate sections. Casters and syracuse dishes were used for most observations with a binocular microscope and these were kept covered to prevent shifting of the pH ; when controls were used they were kept at the same number of foot candles of illumination (Weston photometer) as the experimental dishes, since light has an accelerating effect on metamorphosis.

Results

An excess of the chlorides of Na, K, Ca and Mg in sea water. General observations on the effects of concentrating these chlorides in sea water have already been published (Lynch, 1947, 1949a). During the summers of 1951 and 1952 experiments on the effects of an excess of each of the four chlorides were carried out by using only isotonic solutions in sea water, in proportions of 80/20, 50/50 and 20/80 cc. For magnesium and potassium the results were essentially the same as those reported (Lynch, 1949a). Metamorphosis was inhibited at a pH of 7.1, that of the original mixtures, and at 8.0 (raised by NaOH in magnesium mixtures and by

Table I

The effects of an excess of NaCl on the motility of larvae

Larvae in 80 cc. isotonic NaCl/20 cc. sea water pH = 8.1 (NaOH)				Larvae in normal sea water on the same day			
No. of larvae	No. active in hours			No. of larvae	No. active in hours		
	5	12	24		5	12	24
70	50	0	0	34	16	5	1
115	47	3	0	37	22	2	1
48	16	0	0	59	24	7	1
8	5	0	0	29	14	4	1
12	8	0	0	12	5	4	0
253		3 = 1.1%		171		22 = 12.2%	

KOH in those containing an excess of potassium). The prolonged swimming, formerly observed in the magnesium mixtures, was evidently due to the hydronium ion, since it does not occur at a pH of 8.0 and experiments with sea water acidulated by HCl have shown a considerable lengthening of the natant phase. Since the almost immediate metamorphosis observed in the 50/50 mixtures of 1 N NaCl and $CaCl_2$ could have been effected by the hypertonicity of these solutions, especially the former, the experiments were repeated with isotonic NaCl and $CaCl_2$. A high osmotic pressure was undoubtedly the cause of accelerated metamorphosis in the case of sodium chloride, for proportions of 80 cc. of isotonic NaCl/20 cc. of sea water did not hasten setting; in fact, metamorphosis did not occur in some experiments. In general, only larvae that attached to the surface or to the photonegative

TABLE II

The normal duration of the natatory phase in sea water

No. of larvae	No. inactive in hrs.			
	4	8	12	24
73	71	—	73 (100%)	73
39	28	—	33 (87%)	39
56	21	—	35 (62%)	56
86	7	28	38 (44%)	73
138	84	102	123 (90%)	129
109	4	14	66 (60%)	109
34	—	—	29 (85%)	33
37	—	—	35 (94%)	36
59	—	—	51 (86)	58
29	—	23	25 (86)	28
12	—	3	8 (66)	12
14	—	10	13 (93)	14
686			529	

Average number inactive at 12 hrs. = 77%. Sigma = 16%.

side of the dishes containing an excess of NaCl metamorphosed normally. The natatory period, however, was abbreviated as reported for the normal solutions (see Table I). On the other hand 80 cc. of isotonic $CaCl_2$/20 cc. of sea water gave results essentially identical with those described for normal solutions, but metamorphosis occurred almost immediately only when the pH had been raised to 8.0 ($CaCO_3$, NaOH, KOH and borate buffers gave similar results). The reverse proportions, however (20 cc. of $CaCl_2$/80 cc. of sea water), failed to show any statistically significant difference between the behavior of larvae in these media and that of the controls. The cause of the observed prolongation of the natatory period in solutions having the same proportions of sea water and 1 N $CaCl_2$ remains obscure. It seems unlikely that it was mere coincidence, for larvae are rarely active at 24 hours in sea water (*cf.*, Table II), unless the pH is lowered to about 6.5, and experiments with isotonic solutions gave no indication of such a drop in pH. (Table III shows that many larvae are active at 24 hours in these solutions at a low pH.) These studies have also shown that hypertonic solutions ac-

celerate metamorphosis, regardless of whether the osmotic pressure is raised by ions of salts or by molecules of sugar. In former experiments with solutions made hypertonic by adding sucrose to sea water, the osmotic pressure had not been raised sufficiently high to give accelerating effects. (Hastening of setting does not occur if the freezing point depression is much below 2.73.)

TABLE III

The natatory period of larvae in 80 cc. of sea water and 20 cc. $CaCl_2$ at pH = 6.4

Experimental medium		Controls (same days; pH = 6.4)	
No. larvae	No. active at 24 hrs.	No. larvae	No. active at 24 hrs.
84	17	88	2
81	6	44	1
76	6	89	5
185	25	58	0
145	41	64	4
101	25	69	0
41	4	12	2
31	6	16	2
744	130 = 17.4%	440	16 = 3.6%

Three-ion combinations. These mixtures were made by omitting one of the ions from the following proportions used by LeFevre (1948) in making artificial sea water from isotonic solutions: 500 cc. of NaCl, 10 cc. of KCl, 15 cc. of $CaCl_2$, 2 cc. of $NaHCO_3$, 40 cc. of $MgCl_2$ and 15 cc. of $MgSO_4$. Van't Hoff's (beta) solutions, however, were used for calcium-free media and $MgSO_4$ was omitted in all solutions containing calcium (because of precipitation) and its place was taken by 15 cc. of $MgCl_2$. Experiments were performed with solutions in which the missing ions were replaced by an equal volume of isotonic sucrose and with mixtures without sugar but having the three ions in the same ratio as in artificial sea water. The presence or absence of sucrose seemed to have no effect, although it was found that metamorphosis was inhibited in solutions containing 80 cc. of sucrose/20 cc. of sea water at a pH of 7.8 (glycine buffer). This procedure of making three-ion media is open to the objections summarized by Heilbrunn (1943, p. 455) and the absence of sulphate may have had an independent effect. (For the effects of an absence of sulphate on echinoderm animal-halves treated with lithium, see Needham, 1950, p. 492.) Larvae were placed for a minute or two in 15 cc. of the three-ion medium before being transferred to another dish of the same solution. Generally three or four transfers were made to minimize contamination during seeding with larvae. Nevertheless, the term "ion-deficient media" is preferable to "ion-free" since a slight amount of contamination is unavoidable. The pH was raised to 7.8–8.0 by NaOH and KOH in all experiments.

In all except Mg-free solutions metamorphosis failed to occur. Results were quite similar in Na-free, Ca-free and K-free media. In all of these the second stage of metamorphosis, migration of tissue from the pallial furrow and subsequent pushing of the ciliated covering towards the basal end of the larvae, could

be observed; but the first stage, eversion of the internal sac, failed to occur. This may not be quite true of larvae in Na-free media, for the internal sac protruded from the organisms while swimming but failed to attach them to the substrate. A great elongation of the apical end accompanied by a migration of ciliated covering towards the basal region caused the long axis of the larvae to be lengthened and a prominent bulge to appear in the middle where the ciliated tissue had accumulated in the Na-free media. After an hour and a half the larvae became spherical again and the cilia seemed to disintegrate eventually. Organisms in Ca-free van't Hoff's solution failed to show any protrusion of the internal sac and eventually shed their outer ciliated coverings into the surrounding medium exactly like those placed in an excess of $MgCl_2$ (Lynch, 1949a). The larvae exhibited neither the usual light reactions nor the back-and-forth swimming of the controls. Highly concentrated in the center of the dish, these larvae confined their movements to narrow circumscribed areas, consisting of either circular counterclockwise swimming or rotation about a fixed point. By 6–8 hours these movements had ceased but ciliary action continued. (The latter was most prolonged at a pH of 6.3.) By 24 hours cytolysis was pronounced in larvae left in this medium. Motility was much better preserved in Ca-free than in Na-free media, for in the latter there was scarcely any movement at 1½ hours. In K-free media the behavior was essentially like that described above, except that adhesive fluid was gradually shed from the internal sac and congealed behind the larvae while they were still swimming. (This phenomenon is not at all uncommon in experimental media, for it occurs in sea water when the pH is lowered to 7.0 by McIlvaine buffer and in other cases to be described.) Again, in K-free solutions the behavior of the larvae strikingly resembled that of organisms placed in an excess of magnesium chloride.

Metamorphosis occurred only in Mg-free media within a pH range of 7.4 to 8.0 (raised by NaOH in the latter). Setting was considerably accelerated, having been completed in nearly all larvae within a period of 30 minutes to 3 hours. (The normal duration of the natatory period of larvae in sea water is variable, sometimes occurring within two hours and occasionally enduring in a few larvae for 24 hours; *cf.*, Table II. Several trials have shown that by 12 hours $77\% \pm 5$ have metamorphosed.) When metamorphosed larvae were transferred from the Mg-free media to sea water after 3 hours they reached the polypide stage by 24 hours; those left in the original solutions elongated somewhat but did not reach the condition at which stolons are organized into tripod-shaped structures for anchorage, which usually occurs about 8 hours after metamorphosis. All adhered rigidly to the substrate. The first reaction of larvae placed in Mg-free media was one of complete quiescence; then natatory movements were gradually resumed. Many of the organisms emitted small amounts of adhesive material from the internal sac shortly after contact with solutions at a pH of 7.4, that of the original mixture. A deficiency or absence of magnesium had an effect opposite to that of an excess of the ion.

Two-ion combinations. Normal motility of bryozoan larvae can be maintained for an amazingly long time when only two ions are present. (1) In 20 cc. of isotonic NaCl/10 cc. of $MgCl_2$ (pH raised to 8.0 by NaOH) the larvae remained motile for 6–8 hours after three transfers. Again the ciliated covering migrated towards the basal region of the larvae and these organisms lost their reaction to

light, gathering in enormous numbers in the center of the dish. In fact, an unusual protrusion of the apical organ and migration of ciliated covering towards the basal end, or at least the equatorial region, of the larvae seems to be a characteristic reaction when any one of the four ions of sea water is missing from the media. (2) When the proportions were reversed (20 cc. of $MgCl_2$/10 cc. of NaCl at a pH of 8.0) motility was maintained for as long as 10 hours and typical magnesium effects were observed: absence of light reactions and emission of tissue from the pallial furrow. (3) Similar phenomena occurred in 15 cc. of $CaCl_2$/55 cc. of $MgCl_2$ and motility endured for at least 4 hours after three transfers. This is surprising in view of the fact that natatory movements are extremely feeble by 1½ hours in three-ion Na-free solutions. Evidently the absence of potassium favors ciliary action, for it is more injurious to the cilia of the larvae than any other salt except copper when an excess is added to sea water. Likewise magnesium seems to be necessary for natatory movements of long duration, for in Mg-free three-ion combinations only feeble movements persisted for three hours; in solutions of 20 cc. NaCl/10 cc. of $CaCl_2$ (pH = 8.0 by NaOH) the larvae became immobilized almost immediately and began rotating about a fixed point, a movement that immediately precedes metamorphosis under normal conditions, by means of their vibratile flagella. (The latter form a tuft of long hair-like structures at the apical end of the median furrow or lateral groove; movements of these flagella persist long after ciliary action has ceased.) Great care must be taken to separate ionic effects from those due to the pH of the medium, since larvae in sea water acidulated to a pH of 5.8–6.0 (HCl) may continue swimming for 54 hours or longer, even though the pH will have shifted to 7.0 during 24 hours. Metamorphosis did not occur in any of the two-ion combinations after two transfers, although an atypical kind of setting was observed in 20 cc. of NaCl/10 cc. of $CaCl_2$. Although the larvae did elongate somewhat when removed to sea water after a short exposure to this medium, repeated observations could not determine whether this was a true metamorphosis or not. It is difficult to distinguish the chaotic condition of metamorphosis from rapid cytolysis that generally occurs under these conditions. Even the small amount of sea water added during seeding allowed normal setting in 20 cc. of $MgCl_2$/10 cc. of NaCl; larvae on the surface formed zooids with tripod-like stolons for anchorage. (It is interesting to note in passing that this is characteristic of *B. flabellata,* whereas *B. turrita* always develops four symmetrically arranged stolons, each branching dichotomously at its distal end into two branches.)

Neutral red in sea water. In the following experiments one drop of 0.1% aqueous solution of neutral red was added to 10, 30, 50, 100, 150 and 200 cc. of sea water (range of concentration = 0.001–0.00005%; 1:100,000–1:2,000,000 parts). The pH of the highest concentration was 7.8–8.0 and the freezing point depression was 1.71. Both *B. turrita* and *B. flabellata* were employed during the summer of 1951, but only the latter in 1952; the controls and the experimental organisms were always of the same species and dishes containing them were placed in diffuse light of 75 foot-candles. Temperatures varied from 25–29° C. Larvae were transferred once from a test solution to another of equal concentration to minimize the dilution which occurred during seeding.

Two striking effects were caused by the neutral red media. First, the photonegative response, which generally takes place in sea water at two–three hours,

had set in by an hour in all solutions; and in most cases the change from positive to negative phototropism began within ten minutes after placement of the larvae. These organisms were also more intensely negative than the controls and frequently attached *en masse* at a spot on the periphery of the dish farthest from the source of light. Secondly, metamorphosis was accelerated in solutions containing one drop/10, 50 and 100 cc. of sea water (concentrations = 0.001–0.0001%), but dilutions greater than 0.0001% had no appreciable effect. The degree of acceleration appeared to be somewhat roughly proportional to the concentration; except in media containing one drop/10 cc., the duration of the natant phase varied with that of the controls, being shortest on days when the latter metamorphosed more quickly. In solutions containing one drop/10 cc. of sea water, the free-swimming period was about an hour with very little deviation on either side. In the other solutions found to be effective the extreme duration of the natatory period was four hours; at this time only a few larvae were still active, whereas the majority of the controls were motile at 12 hours.

Subsequent development depended upon whether the larvae were left in the neutral red solution or transferred to sea water after metamorphosis. The results of various concentrations may be summarized as follows: (1) Larvae in the most concentrated solution (1/10) had an elongation equal to that of the controls by 24 hours, but actual development was better when the larvae were transferred to sea water after metamorphosis had been induced precociously, for in the latter case differentiation sometimes exceeded that of the controls. In one experiment in which larvae were allowed to remain in the neutral red solution, about ⅔ had become decidedly stained and at 24 hours these had formed normal zooids as well advanced as the controls except that polypide formation was somewhat inferior; the other ⅓ were free from dye and had not metamorphosed. (2) In solutions of one drop/50 cc. of sea water development was inferior to that of the controls in three out of four experiments in which the larvae remained in the neutral red solution for 24 hours; differentiation was normal, however, when the organisms were removed to sea water after metamorphosis. In at least some cases of poor zooid formation the effect can be attributed to a loss of fluid from the internal sac while the larvae were still swimming; this fluid congealed to form threads which resembled those that had been observed when organisms were placed in sea water maintained at a pH of 7.0 by potassium phosphate McIlvaine buffers, except that the threads formed a network in the latter. Since the holdfast material forms the zooecial wall of the zooid, its deficiency would result in poor development. (For the contribution of larval parts to the adult organism the monograph of Corrêa (1948) on the embryology of *Bugula flabellata* should be consulted.) (3) In dilutions as great as one drop/100 cc. development was equal to that of the controls even when the larvae were not transferred to sea water. (4) In solutions found to be ineffective in appreciably accelerating metamorphosis, development was variable (as it often was in the controls), and in general it seemed to be no different from that of larvae in sea water.

Staining reactions of neutral red. The apical half of the pyriform larvae (containing the crown of rigid cilia) stained very lightly, whereas the basal half (containing the internal sac) stained heavily, the dye being concentrated in definite spots causing the larvae to have a mottled appearance. Staining was generally

pronounced within 20–30 minutes after immersion in the most concentrated media (one drop of neutral red/10 cc. of sea water and one drop/30 cc.). During the process of metamorphosis, material from the pallial furrow (lacking cilia) did not take the dye; and as this material migrated slowly to the basal end of the larvae, the dye gradually became concentrated in granular spots beneath it in this region. The ciliated covering, which is gradually pushed inside the metamorphosing larva by advancing material from the pallial furrow, did not take the stain. This could be determined under abnormal conditions (Ca-free solutions) in which the ciliated covering was shed into the surrounding medium. Holdfast material was likewise largely colorless except for a few granules. After metamorphosis had been completed, the zooecial wall of the first polypide was almost free from stain except for a few red granules scattered here and there but especially concentrated at the distal end where the lophophore would form later. The former body of the larva, located about a third of the distance between the attached end and the free lophophore region, was deeply stained. In later development the polypide, containing the digestive tract, was deeply stained within the zooecial wall. Some of the dye went into the bud for the second zooid of the colony, again concentrating at the tip where the lophophore would form. Buds for the third zooid generally remained free from stain. The tentacles as well as the stolons, except at the distal ends of the latter, were colorless.

Neutral red in three-ion combinations. These media were made as described above and one drop of neutral red/10 cc. of the three-ion media was added. Except for a decrease in motility the behavior of larvae in these media was essentially the same as in these solutions without dye. Neutral red, although a powerful accelerator of metamorphosis, was ineffective in bringing about this phase of the life cycle of the larvae. In fact, there have been no cases encountered so far in which neutral red has effected metamorphosis in solutions that inhibit the process. (This is true also of a pH below 6.0.) The only significant differences in the K-free, Ca-free and Na-free media were: (1) In K-free solutions minute amounts of adhesive material were lost and as the ciliated covering was shed, it became entrapped in this fluid; this did not occur in Ca-free and Na-free solutions, for in the former the ciliated cells were shed into the medium and in Na-free there was no noticeable loss of ciliated covering. (2) Larvae first placed in the ion-free media and then removed to sea water within an hour showed obvious elongation only in the case of Na-free media; there was a very slight elongation of larvae placed in K-free media, but the organisms were only one-fourth normal size (probably because of the loss of adhesive fluid). Larvae in these media failed to show the violent photonegative response of the controls (the same concentration of dye and sea water), generally being quite indifferent to light; in general, the organisms behaved remarkably like those in sea water containing an excess of magnesium chloride. The absence of any one of the ions, except perhaps Na, definitely inhibited metamorphosis, probably by accentuating the anaesthetizing effects of magnesium.

Larvae in Mg-free media exhibited the most striking effects, for a deficiency of the ion accentuated the accelerating properties of neutral red, metamorphosis taking place within 15–30 minutes. Organisms that remained in this solution had slightly elongated by 24 hours, whereas those removed to sea water developed

normally but at a somewhat retarded rate, having developed polypides only by 38 hours. (Polypides are normally recognizable before 24 hours.) All these larvae attached rigidly to the substrate. In view of the inhibiting effects of magnesium, these results might have been anticipated.

Neutral red in sea water at reduced temperatures. In previous experiments it had been observed that cold sea water inhibited metamorphosis of the larvae of *B. neritina* (Lynch, 1947). If neutral red accelerates metamorphosis by activating larval enzymes, a reduction of temperature should reduce the effect. To verify this hypothesis two test solutions were made by adding one drop of neutral red/10 cc. of sea water; the temperature of one of these was maintained at 4° C.

TABLE IV

The effects of concentration of neutral red on the duration of the natatory period

I. Larvae in one drop of 0.1% aqueous soln. of neutral red/10 cc. of sea water				II. Larvae in one drop of 0.1% aqueous soln. of neutral red/30 cc. of sea water			
No. larvae	Number unmetamorphosed in hours			No. larvae	Number unmetamorphosed in hours		
	1	2	3		1	2	3
19	2	2	0	45	19	4	1
27	1	0	0	75	64	6	0
30	2	0	0	91	62	4	1
27	3	0	0	19	13	1	1
25	1	0	0	53	39	25	21
45	9	0	0	42	20	6	1
84	9	9	0	298	188	43	4
65	19	6	0	303	243	19	1
42	13	2	0	42	14	8	1
85	1	0	0	30	13	10	3
52	0	0	0	27	14	10	3
22	7	0	0	16	15	8	7
23	3	0	0				
547	70 = 12.8%			1041	704 = 67.2%		

and the other was kept at 12° C. The motility of larvae immersed in these media was greatly reduced by half an hour and the organisms all settled to the bottom exhibiting neither positive nor negative reactions to light. Metamorphosis did not occur at the usual time for larvae in this concentration of neutral red, for only a few had begun the process by an hour and fifteen minutes and these did not attach rigidly as they did at room temperature. The second stender dish (12° C.) was allowed to warm at this time, and by an hour and forty-five minutes only 20% had metamorphosed; these had their internal sacs everted upwards. By four hours only 50% had metamorphosed and these organisms showed no elongation by 24 hours; the controls (larvae in the same concentration of neutral red and sea water at room temperature) had metamorphosed by an hour. Cooling the medium, therefore, inhibited metamorphosis even after the solution came to room temperature.

Neutral red in isotonic solutions. These media consisted of one drop of neutral red/10 cc. of isotonic solutions of the four chlorides. Metamorphosis was not observed in any of them and motility was greatly reduced, never enduring for more than half an hour. Calcium chloride had the most deleterious effect, causing the cilia to dissolve and bringing about cytolysis of the larvae to a marked degree. In the potassium solution ciliated tissue was shed just as it was in sea water containing an excess of this ion (Lynch, 1949a). In the sodium chloride solution the larval covering also disintegrated instead of being pushed inside by tissue from the pallial furrow which had begun to migrate towards the basal end. Magnesium chloride merely anaesthetized the larvae causing the cilia to stand out like a circlet surrounding them. These results were similar to those obtained by using isotonic solutions without neutral red. It is interesting to note that Bradway (1936) had found isotonic calcium chloride to be the most deleterious of all the chlorides in its effect on ascidian larvae; magnesium chloride was relatively non-toxic and inhibited metamorphosis.

Methylene blue in sea water. Methylene blue, though much less effective in accelerating metamorphosis than neutral red, gave results similar to those just described. Only two concentrations were used, one drop of 0.1% aqueous solution/10 cc. and 50 cc. of sea water. The pH was 7.6 and the F.P.D. was 1.71. Motility was considerably greater than in similar concentrations of neutral red and the photo-negative response not only occurred later but was also less pronounced. Although the blue dye was not absorbed as readily nor as extensively as neutral red, the staining reactions were similar, the apical end being almost colorless; whether this was caused by a failure of this region to absorb the dye or by a reduction process is not known. More larvae were geo-negative in the methylene blue media than in those containing neutral red. Except for larvae transferred to sea water after metamorphosis, zooid formation was rather poor in the more concentrated solution; those that attached to the surface, however, developed normally even when they were not transferred to sea water, but geo-negative settings did not.

Discussion

Several explanations of the *modus operandi* of metamorphosis have been offered in the past by various investigators. Thirty years ago Huxley (1922) suggested that unfavorable environmental conditions can bring about the changes, generally cataclysmic, that reduce a free-swimming larva to a state of morphological chaos followed by a constructive phase of differentiation and growth. After comparing similarities between normal metamorphosis in Echinus plutei and dedifferentiation in hydroids and in the ascidians, *Clavelina* and *Perophora,* he concluded that the two processes are essentially similar and predicted that toxic agents would induce precocious metamorphosis in echinoderm larvae.

Certainly copper, $HgCl_2$, $ZnSO_4$, distilled water, isotonic salt solutions, starvation and vital dyes would seem to affect larvae adversely. (For the relative toxicities of neutral red, methylene blue and Janus green, see Child and Rulon, 1936.) Although there is much logic in the proposition of Huxley (1928) that Echinus larvae metamorphose when their increasing weight causes them to sink to the bottom away from the more favorable conditions of food and oxygen at the surface (and this idea would be applicable to ascidians, which become geo-positive

just before setting), the same explanation cannot be used for bryozoan larvae that frequently attach to the surface. Although the internal environment might become unfavorable because of the accumulation of toxic products of metabolism or starvation (since an alimentary canal is absent), the external environment is apparently unchanged just before setting. The hypothesis that an unfavorable environment causes metamorphosis is an unsatisfying one from a chemical viewpoint, since it does not explain why metamorphosis occurs under adverse conditions.

At first sight the hypothesis that larval enzymes are involved in metamorphosis seems to have much in its favor. The observations already presented, as well as those of Bradway (1936) and Zhinkin (1938), on the effects of vital dyes in accelerating metamorphosis would seem to indicate that a stimulation of proteolytic enzymes is somehow involved. On the other hand, Glaser and Anslow (1949) proposed as a tentative hypothesis that copper inactivation of larval enzymes is the "key to morphological disintegration" characteristic of the disruptive phase of ascidian metamorphosis. How, then, can two viewpoints, one of stimulation of larval enzymes and the other of inactivation, be reconciled? It may be that some agents, such as copper, can inactivate certain enzymes, perhaps succinic-dehydrogenase systems, that are necessary for larval life; others may stimulate autolysis. The same net result—larval dedifferentiation—could presumably be attained by stimulating proteolytic enzymes or by inactivating larval oxidation-reduction systems. The failure of bryozoan larvae to develop in copper solutions capable of accelerating metamorphosis (Lynch, 1949a) should be expected, according to the enzyme hypothesis, for copper would apparently be disastrous not only to the larvae but to the development of adult structures as well.

The hypothesis that autolysis initiates metamorphosis and that this process is accelerated by vital dyes leaves much to be desired. Huxley (1922) could find no evidence for believing that cytolyzing enzymes are a necessary hypothesis for explaining the resorptive dedifferentiation that occurs in hydroids and in *Perophora*. Grave (1935) likewise could find no support for Berrill's theory (1929) that phagocytosis of the tail of ascidian tadpoles is the initiator of metamorphosis. If factors causing the acceleration of bryozoan metamorphosis are listed opposite those bringing about retardation, it seems possible that the "colloidal theory of calcium release" may explain most of the facts. Accelerating agents may conceivably effect metamorphosis by a direct action on protoplasmic viscosity.

Accelerators of metamorphosis:	Inhibitors of metamorphosis:
Heat (Marcus, 1926; Lynch, 1949b)	Cold (Lynch, 1947)
Hypertonic solutions (Lynch, 1949a)	Hypotonic solutions (Lynch, 1947)
Absence of magnesium	Excess of magnesium (Lynch, 1949a)
Moderate amount of diffuse light (Grave, 1930)	Absence of light (Lynch, 1949b)
	Excess of potassium (Lynch, 1949a)
Copper (Lynch, 1949a)	Absence of Na, K or Ca in three-ion combinations
Large excess of calcium	
Vital dyes	A pH below 6.0 (Lynch, 1949a)

It seems more than merely coincidental that inhibitors of metamorphosis, such as cold sea water, an excess of magnesium or potassium, are also agents that have an anaesthetizing effect. Interpreted in terms of the calcium-release theory, in-

hibition of metamorphosis may be merely a kind of anaesthesia, reducing the activity of larvae to a minimum and thus forestalling the unfavorable effects of starvation. (For the narcotizing effects of magnesium and potassium see Heilbrunn, 1943, p. 520.) The absence of calcium, at least, should accentuate the anaesthetizing effects of magnesium (Heilbrunn, 1943, p. 531); what effects the absence of sodium or potassium have on protoplasmic viscosity are not well known. It seems logical to attribute the inhibitory effects of hypotonic sea water on the larvae of *B. neritina* to a decreased protoplasmic viscosity caused by absorption of water. (If, however, sea water is diluted by 50% with distilled water and larvae are placed in it for ten minutes and then returned to sea water, metamorphosis and zooid formation take place.) Accelerating agents, on the other hand, rather closely parallel those that stimulate protoplasm or cause a clotting of the interior of cells. Thus, hypertonic solutions, which accelerate metamorphosis, are known to cause calcium release from the cortical layer resulting in a concomitant increase of the viscosity of the inner protoplasm. Since light can also release calcium and increase the viscosity of the interior protoplasm (Alsup, 1942), an absence of light may presumably have the opposite effect—hence the inhibiting effects of darkness. (It is interesting to note that Knight-Jones, 1951, observed that the trochophores of *Spirorbis* behave like the larvae of *Bugula* in darkness; both attach to the surface film predominantly. In fact, the pre-fixational activities of *Spirorbis* are so remarkably similar to those of *Bugula* that the parallel suggests that behavioral patterns may be preserved through evolutionary changes.) The action of vital dyes may be interpreted, perhaps, as direct effect on protoplasmic viscosity rather than an indirect one by activating proteolytic enzymes. The studies of Alsup (1941, 1942) on the photodynamic action of rose bengal and eosin on protoplasmic viscosity of the interior of cells, increasing it in the presence (but not in the absence) of light, suggest that neutral red and methylene blue may have effects similar to the dyes he used. All these dyes seem to affect protoplasm in much the same way. The writer found eosin effective in initiating metamorphosis, but it was even less potent than methylene blue, and Hassett (1941) found that rose bengal, eosin, neutral red and methylene blue all had similar effects on the light responses of *Peranema tricophorum*. Whether vital dyes affect the protoplasm directly or only indirectly via an enzyme system is problematical, for Heilbrunn (1943, p. 538) stated that "the clotting of protoplasm may well be related to some proteolytic enzyme action." At present it is generally believed that dyes with a photodynamic action affect exposed —SH groups of the protein molecule. (*Cf.*, Calcutt, 1951.) The observations of Angerer (1937, 1942) that copper chloride (an accelerator of metamorphosis) increases the viscosity of sea urchin eggs and of the protoplasm of *Amoeba dubia* may indicate that copper also has a direct effect on larvae rather than an indirect one by inactivating larval enzymes. At any rate, the hypothesis of Glaser and Anslow (1949), emphasizing the role of copper in inactivating larval enzymes, seems to be inadequate, since it fails to take into consideration the host of other substances that accelerate metamorphosis.

There is, therefore, some cumulative indirect evidence for the hypothesis that anaesthetic agents inhibit metamorphosis and that factors causing a release of calcium accelerate it; if it is a tenable one, further experiments should show a general effect of all narcotizing agents. (It is interesting to note that Grave

(1935) observed a narcotizing effect of something in the medium when adrenalin was used in experiments on ascidian metamorphosis; attachment was inhibited.) If coagulating agents can induce metamorphosis, thromboplastic-like substances should hasten the process and heparin should inhibit it. Harding (1951) found that injury substance from minced frog muscle initiated cell division parthenogenetically in *Arbacia* eggs. This substance, according to Heilbrunn *et al.* (1936), is apparently a thrombin-like material. Heilbrunn and Wilson (1949, 1950) found that heparin, a bacterial polysaccharide acting like heparin and dicumarol, inhibited cell division in the eggs of *Chaetopterus*. None of these substances has been tried for inhibiting effects on bryozoan larvae, but injury substance made from frog muscle according to the procedure outlined by Harding (1951) not only failed to accelerate metamorphosis but actually inhibited it. Seven or eight trials with this substance, extracted from frog muscle on four or five occasions, all gave negative results. At a pH of 4.5, found to be effective by Harding (1951), the larvae became immobilized immediately and merely disintegrated after a few hours. Since metamorphosis is inhibited at a pH as low as 4.5, injury substance was tried within a pH range of 5.7, 7.6 and 8.3. (KOH and borate buffers were used in raising the pH of the originally acid injury substance) in combinations ranging from 3 cc. injury substance/7 cc. of sea water, the reverse proportions and other mixtures. In all cases, from pH 5.7–8.3 the results were similar. The larvae gradually lost adhesive material from the internal sac, became stuck to one another, eventually disentangled themselves and continued to swim for 8–10 hours. Metamorphosis could not be induced even when neutral red was subsequently added to these media after five hours exposure. Results similar to these were obtained with the whole blood of frogs added to sea water. In some of these experiments with thrombin-like substances the internal sac ruptured completely without being everted; but the process was mechanical and not one in which the larvae actively participated. In all cases the entire medium in which larvae were placed became extremely cloudy because of congealed adhesive material. Yet the fact that these substances did cause emission of attachment fluid might lead to the supposition that clotting agents in exactly the right proportions might prove effective in accelerating metamorphosis. The theory of calcium release would seem to offer some explanation for the accelerating effects on ascidian larvae caused by an extract of muscle tissue of rabbits killed by x-rays (Bertholf and Mast, 1944) and by whole thyroid gland (but not thyroxine), mantle tissue, etc. (Grave, 1935). In fact, Grave stated (1935, p. 288) that, "If muscle tissue of whatever origin should prove to contain an accelerating substance, an explanation might be afforded for the exceptional results of experiment 191, in which an extract of mantle and foot tissue of a snail . . . induced 100% metamorphosis in a group of larvae of *Polyandrocarpa*. . . ." Although Grave considered that the effective agent in muscle might be an endoenzyme or ferment, it seems more likely, in view of the recent experiments of Heilbrunn *et al.* (1946), that a thromboplastic-like substance may be involved. Like carcinogens and like parthenogenetic agents, factors influencing metamorphosis are many and varied, apparently having little or no relation to one another. Only extensive experiments in the future can bring the complicated process of metamorphosis nearer to an acceptable solution.

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Summary

1. In calcium-free van't Hoff's solution and both Na-free and K-free mixtures having the other ions in the same proportion as in sea water at a pH of 7.1–8.0, the larvae of *Bugula flabellata* behaved exactly like those in sea water containing an excess of $MgCl_2$. Phototropic reactions were lost, ciliated tissue was shed as material migrated basally from the pallial furrow and metamorphosis did not occur.

2. In isotonic Mg-free solutions (pH = 8.0) metamorphosis was greatly accelerated, fixation occurring within 30 minutes to 3 hours in the majority of larvae; these organisms developed well-formed zooids in 10 hours. (Normally only about 77% of the larvae metamorphose by 12 hours.)

3. Neutral red, methylene blue and, to a lesser extent, eosin accelerated metamorphosis. Neutral red, the most potent of the three, was effective in concentrations of 1 : 1,000,000 parts of sea water; methylene blue caused acceleration in concentrations of 1 : 500,000 parts. At a concentration of 1 : 100,000 parts of neutral red the average duration of the natatory period was about an hour.

4. Metamorphosis failed to occur in Ca-free, Na-free and K-free media to which neutral red had been added in proportions of 1 : 100,000 parts. The same concentration of neutral red in isotonic Mg-free media (pH = 8.0) had a greater accelerating effect than when the dye was added to sea water in the same proportions. A reduction of temperature to 4° C. to 12° C. of sea water containing neutral red in parts of 1 : 100,000 antagonized the accelerating effects of neutral red. The staining reactions of neutral red and the problems involved in the study of metamorphosis are also discussed.

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